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Evaluation of the wound healing property of *Boesenbergia longiflora* rhizomes

Teeratad Sudsai [a,b], Chatchai Wattanapiromsakul [a], Titpawan Nakpheng [c], Supinya Tewtrakul [a,b,*]

[a] Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla 90112, Thailand
[b] Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Songkhla 90112, Thailand
[c] Drug Delivery System Excellence Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

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ABSTRACT

Ethnopharmacological relevance: The rhizomes of *Boesenbergia longiflora* (Wall.) Kuntze (Zingiberaceae) have been traditionally used for treatment of inflammatory bowel disease, ulcerative colitis, aphthous ulcer and abscess by decoction with alcohol.
Aim of the study: The rhizomes of *Boesenbergia longiflora* were carried out to investigate for anti-inflammatory and wound healing activities in order to support the traditional use.
Material and methods: The ethanolic extract of *Boesenbergia longiflora* and its fractions were tested using relevant *in vitro* anti-inflammatory and wound healing assays. For the *in vitro* studies, murine macrophage RAW264.7 cells and mouse fibroblast L929 cells were assessed for anti-inflammatory and fibroblast stimulatory activities, respectively. *In vivo* anti-inflammatory activity was determined by carrageenan-induced rat paw edema model as well as acute toxicity estimated by the up-and-down method in mice.
Results: The present study has demonstrated that the ethanolic extract of *Boesenbergia longiflora* rhizomes possesses a potent anti-inflammatory and wound healing activities. Among the isolated fractions, the $CHCl_3$ fraction showed potent anti-inflammatory effect through nitric oxide inhibitory activity (IC_{50}=5.5 μg/ml) and reduction of carrageenan-induced rat paw edema (ED_{50}=222.7 mg/kg), whereas this fraction exhibited wound healing property via fibroblast migration on both day 1 (77.3%) and day 2 (100%) as well as enhanced collagen production (187.5 μg/ml) at concentration of 3 μg/ml, compared to that of the controls, 39.4% for fibroblast and 60.8 μg/ml for collagen, respectively. The anti-inflammatory mechanism of the $CHCl_3$ fraction is found to suppress the iNOS and COX-2 mRNA expression.
Conclusion: The scientific investigation of wound healing activity of *Boesenbergia longiflora* rhizomes support the Thai traditional uses for treatment of inflammatory bowel disease, ulcerative colitis, aphthous ulcer and abscess. The EtOH extract and $CHCl_3$ fraction exert potential wound healing property through NO inhibition, anti-oxidant effect and stimulation of fibroblast migration and collagen production. The phytochemical screening revealed that the $CHCl_3$ fraction of *Boesenbergia longiflora* rhizomes contains diarylheptanoids, flavonoids and terpenes. The isolation of the compounds responsible for the wound healing effect is now in progress.

1. Introduction

Wound healing process comprises a complex of events which repair the damaged tissue partially or completely depending on the severity of wounding. This process can be characterized by three overlapping phases; inflammatory phase (consisting of hemostasis and inflammation), proliferative phase (consisting of granulation, contraction and epithelialization) and remodeling phase which organized structure with increased tensile strength (Wild et al., 2010). Many factors influence wound healing by impaired and delayed the process of wound repair such as inflammatory and immune responses as well as microbial infection (Houghton et al., 2005). It is well known that when wounding occurs, short-term process of inflammation accompanied by swelling, redness, pain, heat generation and loss of cell function caused by the release of the inflammatory mediators by the

* Corresponding author at: Prince of Songkla University, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Kanchanawanit Street, Songkhla 90112, Thailand.
Tel.: +66 74 288888; fax: +66 74 428220.
E-mail addresses: supinyat@yahoo.com, supinya.t@psu.ac.th (S. Tewtrakul).

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macrophages mainly take the crucial role (Houghton et al., 2005; Tam et al., 2011). During this process, macrophages generate inflammatory mediators such as nitric oxide (NO) to eliminate foreign pathogens, recruit other cells to the infected area and subsequently resolve the inflammation. However, excessive production of NO also attacks normal tissue surrounding the infected area by binding with other superoxide radical and act as a reactive radical which damages normal cell function (Min et al., 2009; Tewtrakul et al., 2009). Thus, the inhibition of reactive radical production is an important consideration in recruitment of fibroblast which is attracted into the site to initiate the proliferative phase of repair or wound healing process (Houghton et al., 2005). The effects of pharmacological agents which modulate the different phases of the wound healing processes can be assessed by *in vitro* experiments and ideally a plant-based remedy should affect at least two different processes before it can be said to have some scientific support for its traditional use (Houghton et al., 2005).

There are approximately 80 species of the genus *Boesenbergia* Kuntze (Zingiberaceae) distributed throughout tropical Asia. Nineteen species of which are indigenous to Thailand, most of the members at wild states, which are not yet to be identified. Local literature survey has pointed out that there are four species including *Boesenbergia longiflora* (Wall.) Kuntze, *Boesenbergia xiphostachya* (Gagnep.) Loes., *Boesenbergia rotunda* (L.) Mansf and *Boesenbergia* sp. that have been medicinally used in Thailand (Chuakul and Boonpleng, 2003; Techaprasan et al., 2006). Only *Boesenbergia rotunda*, known as Krachai, is widely distributed and commonly cultivated for their edible rhizomes in Thailand and Southeast Asia. In addition, *Boesenbergia rotunda* is the only species in the genus *Boesenbergia* that is medicinally important for its panduratin content (Tuchinda et al., 2002; Cheenpracha et al., 2006; Kiat et al., 2006; Techaprasan et al., 2006; Ching et al., 2007; Tewtrakul et al., 2009). *Boesenbergia longiflora* is another related crop under this genus which has a potential for great exploitation on biological properties. The rhizomes of *Boesenbergia longiflora* (synonym: *Curcumorpha longiflora* (Wallich) A. S. Rao & D. M. Verma) have been traditionally used in the treatment of inflammatory bowel disease, ulcerative colitis, aphthous ulcer, stomach discomfort, dysentery and abscess by decoction with alcohol (Delin and Larsen, 2000; Chuakul and Boonpleng, 2003; Kanathum, 2008).

The present study was therefore aimed to evaluate the wound healing effect of *Boesenbergia longiflora* rhizomes by using several *in vivo* and *in vitro* assays. The assessment of the wound healing parameters including anti-inflammatory, anti-oxidant and fibroblast activities were employed for the scientific support.

2. Materials and methods

2.1. Plant material, extraction and fractionation of Boesenbergia longiflora

The fresh rhizomes of *Boesenbergia longiflora* were bought from Chatuchak weekend market in Bangkok, Thailand, in June 2010 and identified by Dr. Charun Marknoi. The voucher specimen (SKP2060200-101) is deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. For the preparation of the extract, the dried rhizomes 5 kg were pulverized and macerated with EtOH at room temperature, four times (6 L × 4). The filtrate was then concentrated under vacuum using rotary evaporator. The extraction yield of EtOH extract of *Boesenbergia longiflora* rhizomes was found to be 11.3% w/w. The EtOH extract (539 g) was partitioned between 95% methanol and hexane, and successively partitioned with chloroform and water. After that, the water layer was partitioned with ethyl acetate (EtOAc). Each partition was evaporated to dryness to obtain the hexane, chloroform, EtOAc and water fractions with the yields of 21.6, 70.5, 0.4 and 4.3% w/w, respectively. The EtOH extract and its fractions were stored at 4 °C until use. The phytochemical study of the $CHCl_3$ fraction was also investigated and they are found to be diarylheptanoids, flavonoids and terpenes. The identification was done by thin layer chromatography (TLC) under UV wavelengths 254 and 365 nm, then spray with anisaldehyde in 10% sulfuric acid and heating the TLC plate at 105 °C. If they are diarylheptanoids or flavonoids, the color is changed to be yellow–brown after heating, whereas the terpenes and steroid derivatives are red–violet. Moreover, the NMR spectroscopic data was also evaluated together with the TLC. From the phytochemical screening, it was indicated that the hexane fraction may contain steroids and terpenes, the choloroform fraction contained flavonoids, diarylheptanoids and terpenes, the EtOAc fraction was found to be flavonoids, whereas the water fraction was sugars. The complete structures of the isolated compounds are now under elucidation.

2.2. Experimental animals

Male and female Swiss albino mice (30–40 g) and male Wistar rats (180–200 g) were used in the experiments. The animals obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat-Yai, Songkhla, Thailand. All experimental protocols were approved by The Animal Ethic Committee, Prince of Songkla University (MOE 0521.11/303). Both Swiss albino mice and Wistar rats were housed in standard environmental conditions with a 12 h light/dark cycle. They were provided *ad libitum* with standard rodent diet and water.

2.3. Cell culture and chemicals

The mouse fibroblast L929 cell line (Chinese Academy of Preventive Medical Sciences, Beijing, China) was cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, Grand Island, NY, USA) and murine macrophage-like RAW264.7 cell line (purchased from Cell lines Services) was cultured in RPMI medium (RPMI, Gibco®, Grand Island, NY, USA) in 75 cm^2 culture flasks at 37 °C in a humidified atmosphere containing 5% CO_2. All mediums were supplemented with 1% penicillin–streptomycin (Invitrogen, California, USA) and 10% fetal bovine serum (FBS; Sigma Aldrich, Missouri, USA). Cells were harvested with 0.25% trypsin–EDTA (Gibco (Invitrogen, California, USA) and diluted to a suspension in a fresh medium for further incubation.

2.4. Anti-inflammatory assays

2.4.1. In vivo anti-inflammatory study

2.4.1.1. Acute toxicity test of Boesenbergia longiflora extract in mice. The 50% lethal dose (LD_{50}) of the EtOH extract and $CHCl_3$ fraction of *Boesenbergia longiflora* rhizomes were estimated by the up-and-down method in mice (Bruce, 1985). The animals were fasted for 6 h prior to dosing. Doses were adjusted by a constant multiplicative factor (*viz.*, 1.5) for this experiment. The dose for each successive animal was adjusted up or down depending on the previous outcome. The crude extracts were dissolved in the cosolvent solution (Tween 80: DMSO: water = 2.5:2.5:95) and orally administered in a single dose by gavage using a stomach tube to both groups of male and female mice. Animal behaviors were observed individually at least once during the first 30 min after administration, periodically during the first 8 h and daily thereafter, for a total of 7 days. The signs of toxicity were observed

including tremor, convulsion, hyperactivity, sedation, grooming, loss of righting reflex, respiratory depression and coma.

2.4.1.2. Carrageenan-induced rat paw edema. The EtOH extract and fractions of *Boesenbergia longiflora* rhizomes were tested in animal model, carrageenan-induced rat paw edema, as described by Winter et al. (1962). Briefly, male Wistar rats (180–200 g) were randomly divided into seven groups. For the EtOH extract and its fractions, 24 animals were divided into 4 doses treatment groups, each comprised of 6 animals. The control group (group 1) was treated with vehicle (10 ml/kg, Tween 80: DMSO: water = 2.5:2.5:95) and the positive control group (group 2) was treated by indomethacin (10 mg/kg). The EtOH extract (group 3) and its fractions (groups 4–7) of *Boesenbergia longiflora* at various doses (50–500 mg/kg) were administered orally to rats for 30 min before subcutaneous injection of 0.1 ml of 1% (w/v) carrageenan in normal saline into the subplantar surface of the right-hind paw. The measurements of paw volume were taken before carrageenan injection and in every 0.5, 1, 2, 3, 4 and 5 h using a plethysmometer (UGO Basile 7140). The volume of hind paw was evaluated for anti-inflammatory activity and was expressed as % inhibition of the hind paw volume. The percentage of inhibition was determined for each experimental group as following equation and the half maximal effective dose (ED_{50}) values were determined graphically ($N=6$).

$$\text{Inhibition } (\%) = [1-(V_t/V_c)] \times 100$$

V_t and V_c are edema volume of hind paw in the sample treated and control group respectively.

2.4.2. In vitro anti-inflammatory study

2.4.2.1. Inhibitory effects on LPS-induced NO production from RAW264.7 cells. RAW264.7 cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 1 μg/ml of LPS together with the test samples at various concentrations (3–100 μg/ml for the extracts and 3–100 μM for the positive controls) and was then incubated for 48 h. NO synthase inhibitor (l-NA), NF-κB inhibitor (CAPE) and non-steroidal anti-inflammatory drugs, NSAIDs (indomethacin, ibuprofen and aspirin) were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final DMSO is 1%).

The NO production by RAW264.7 cells was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent as previously described (Tewtrakul et al., 2009). After 48 h incubation, cells generated NO in the medium, the supernatant (100 μl) was collected and reacted with Griess reagent (100 μl). NO production was measured spectrophotometrically at 570 nm using a microplate reader, PowerWaveX from Bio-Tex Inc. The % inhibition was calculated based on the following equation and IC_{50} values were determined graphically ($N=4$):

$$\text{Inhibition } (\%) = [(A-B)/(A-C)] \times 100$$

A − C: NO_2^- concentration (μM) [A: LPS (+), sample (−); B: LPS (+), sample (+); C: LPS (−), sample (−)].

2.4.2.2. Viability assay of RAW264.7 macrophage cells. Viability of RAW264.7 cells was assayed using MTT colorimetric method after 48 h incubation with various concentrations of test samples. MTT solution (10 μl, 5 mg/ml) was added to the wells and further incubated for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2. Thereafter, the medium was removed, the formazan products made due to dye reduction by viable cells were dissolved using isopropanol containing 0.04 N HCl and the optical density was measured with a microplate reader at a wavelength of 570 nm. The test samples were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control (vehicle-treated) group.

2.4.2.3. Effects on expression of iNOS and COX-2 mRNA. In order to acquire the mechanism for anti-inflammatory activity of *Boesenbergia longiflora* extract, the suppression on mRNA expression of iNOS and COX-2 genes was carried out as previously described by Sae-wong et al. (2009). Briefly, RAW264.7 cells were harvested after 20 h incubation with various concentrations of samples (3–100 μg/ml) and were then isolated to obtain RNA using RNeasy Mini Kit (Qiagen Operon, USA). For first strand cDNA synthesis, RNA from each sample was reverse-transcribed using cDNA synthesis kit (Rever Tra Ace-α kit, Toyobo, Japan). Then PCR analyses were performed on the aliquots of the cDNA preparations for detecting iNOS, COX-2 and β-actin gene expression by PCR kit (Rever Tra Dash, TOYOBO, Japan). The primers for each gene were as follows:

iNOS:	forward primer; 5′-ATCTGGATCAGGAACCTGAA-3′
	reverse primer; 5′-CCTTTTTTGCCCCATAGGAA-3′
COX-2:	forward primer;
	5′-GGAGAGACTATCAAGATAGTGATC-3′
	reverse primer;
	5′-ATGGTCAGTAGACTTTTACAGCTC-3′
β-actin:	forward primer;
	5′-TGTGATGGTGGGAATGGGTCAG-3′
	reverse primer; 5′-TTTGATGTCACGCACGATTTCC-3′

The reactions of cDNA synthesis occurred in a 20 μl volume with RNA solution 11 μl, 5 × RT 4 μl, dNTP mixture (10 mM) 2 μl, RNase inhibitor (10 U/μl) 1 μl, Oligo (dT) 20 1 μl and Rever Tra Ace (reverse transcriptase enzyme) 1 μl for a reaction. The condition for cDNA synthesis was performed at 42 °C for 20 min, 99 °C for 5 min and 4 °C for 5 min. The PCR mixture is consisted of cDNA 2 μl, dH_2O (RNase free water) 85 μl, 10 × PCR buffer 10 μl, forward primer (10 pmol/μl) 1 μl, reverse primer (10 pmol/μl) 1 μl and KOD Dash DNA polymerase (2.5 U) 1 μl, to give a final volume of 100 μl. The condition for PCR was performed for 30 cycles using MJ Mini™ Thermal Cycler (Bio-Rad, USA) as follow; denaturation at 98 °C for 30 s, annealing at 60 °C for 30 s, and extension at 74 °C for 1 min. The PCR products were separated on 1.2% (w/v) agarose gels and were stained with SYBR® Safe DNA staining solution for 30 min and the intensities of the bands were determined under UV irradiation at wavelength 312 nm (Gel Doc model 1000, Bio-Rad, USA).

2.5. Assays relevant to wound healing activity

2.5.1. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging assay

The methodology described by Jitsanong et al. (2011) was used in this study with slight modifications in order to assess the DPPH free radical scavenging capacity. The stock solution (10 mg/ml) of the sample was prepared in DMSO and diluted to concentrations ranging from 1 to 500 μg/ml with an absolute ethanol. The reaction mixture contained 100 μl of samples at various concentrations and 100 μl of 0.1 mM DPPH in an absolute ethanol. The commercial known antioxidants, butylated hydroxytoluene (BHT) and quercetin were used as positive controls. The DPPH solution in the absence of sample was used as a control and the absolute ethanol was used as a blank. The bleaching was measured at 517 nm using a microplate reader after incubation for 30 min in

the dark condition. The percentage of scavenging activity of the sample against DPPH radical was calculated according to the following equation and IC_{50} values were determined graphically ($N=4$):

$$\%\ \text{Inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

$A_{control}$ = Absorbance of control − Absorbance of control blank

A_{sample} = Absorbance of sample − Absorbance of sample blank

2.5.2. Cell proliferation and viability assay using L929 fibroblast

L929 fibroblasts were seeded at 2×10^4 cells/well into 96-well plate in DMEM containing 10% FBS. After 48 h, cells were exposed to different concentrations (1–100 μg/ml) of test samples and were then incubated for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2. The MTT solution (10 μl, 5 mg/ml) was added directly to the medium in each well, and the plate was then incubated at 37 °C for 4 h. All medium was then aspirated and replaced with isopropanol containing 0.04 N HCl, and the optical density at 570 nm was recorded. The percentage of cell proliferation was calculated and compared to a negative control.

2.5.3. Hydrogen peroxide-induced oxidative stress

Hydrogen peroxide (H_2O_2)-induced oxidative stress was determined by measuring cell viability by the MTT assay as previously described by Jitsanong et al. (2011) with some modifications. Briefly, fibroblast L929 cells were seeded in 96 well plates (2×10^4 cells/ml) in DMEM medium containing 10% FBS. After 24 h, cells were treated with different concentrations (1–30 μg/ml) of test samples. After pretreatment with different concentrations for 1 h incubation at 37 °C with 5% CO_2, the cells were co-incubated with 0.5 mM of H_2O_2 for another 24 h. At the end of the incubation, cell viability was determined by the MTT assay.

2.5.4. Migration assay

The migration of fibroblast L929 cells was examined using a wound healing method as previously described by Balekar et al. (2012). Briefly, L929 cells (5×10^4 cells/ml) in DMEM containing 10% FBS were seeded into each well of 24 well plate and incubated at 37 °C with 5% CO_2. After the confluent monolayer of L929 cells was formed, a horizontally scratched with a sterile pipette tip was generated two scratches (left and right) in each well. Any cellular debris was removed by washing with PBS and replaced with 1 ml of fresh medium in the absence or presence of test samples. Photographs were taken two views on the left and right of each well at a 4 × magnification using a microphotograph (Olympus CK2, Japan) on day 0, then plates were incubated at 37 °C with 5% CO_2 and photographs were taken at days 1 and 2. To determine the migration of L929 cells, the images were analyzed using computing software (ImageJ1.42q/Java1.6.0_10). Percentage of the closed area was measured and compared with the value obtained before treatment (day 0). An increase of the percentage of closed area indicated the cell migrations.

2.5.5. Determination of soluble collagen production

The soluble collagen productions are determined according to the method described by Balekar et al. (2012). Fibroblast L929 cells in DMEM containing 10% FBS were seeded at an initial concentration of 2×10^4 cells/ml in a 96 well plate. After 24 h, the culture medium was replaced with a fresh medium containing the test samples at various concentrations (0.3–10 μg/ml) and was then incubated for 48 h at 37 °C with 5% CO_2. Cells without a test sample served as negative controls. After 48 h of incubation, cells generated soluble collagen type I into the medium, the supernatant (100 μl) were collected. The total amount of soluble collagen type I was assayed using the Sircol® Collagen Assay Kit (Bicolor Life Science Assays, Northern Ireland, UK). Briefly, 100 μl of supernatant was mixed with 1 ml of dye solution at room temperature for 30 min. Then the samples were centrifuged at 15,000 × *g* for 10 min to form a pellet of collagen. All supernatant was then aspirated and the soluble collagen was dissolved in 1 ml of alkali reagent. Thereafter, the alkali solutions were transferred to a 96 well plate and the optical density at 540 nm was recorded. The amount of collagen was calculated based on a standard curve of soluble collagen (bovine skin collagen type I standard from American disease free animals).

2.6. Statistical analysis of data

For statistical analysis, all data values were calculated using the Microsoft Excel program and expressed as mean ± S.E.M of four determinations. The data analysis was performed by one-way analysis of variance (ANOVA), followed by Dunnett's test. The p value < 0.05 was considered to be significant.

3. Results and discussion

3.1. In vivo assays relevant to anti-inflammatory activity

3.1.1. Acute toxicity

For the acute toxicity test, the oral LD_{50} of ethanol extract and chloroform fraction of *Boesenbergia longiflora* rhizomes were found to be greater than 2000 mg/kg of body weight in both male and female Swiss albino mice. At the end of study period (7 days), all animals survived and appeared active and healthy. During the day of observation period, the animals were observed no significant sign of toxicity, adverse pharmacological effects or abnormal behavior. This result may indicate that *Boesenbergia longiflora* extract has no acute toxicity.

3.1.2. Carrageenan-induced rat paw edema

Subcutaneous injection of carrageenan into the rat paw produces inflammation resulting from the release of various inflammatory mediators in biphasic. For the first phase, the release of histamine and serotonin begins immediately after injection of carrageenan and diminishes in 2 h, while the second phase the release of prostaglandins, protease and lysosome begins at the end of the first phase and remains through 3 to 5 h (Eddouks et al., 2012). The *in vivo* anti-inflammatory efficacy of EtOH extract and its fractions of *Boesenbergia longiflora* and standard drug, indomethacin, against carrageenan-induced paw edema are shown in Table 1. The injection of carragenan caused localized edema starting at 0.5 h and progressively increased swelling to a maximum volume at 5 h after injection. Paw volumes were measured at 30 min, 1 h, 2 h, 3 h, 4 h and 5 h after induction and the peak activity was observed at 2 h after induction. The EtOH extract and $CHCl_3$ fraction significantly reduced paw volume at 2 h. The percentage of edema inhibition for EtOH extract at 50, 100, 200 and 500 mg/kg doses were 25.3, 37.3, 46.6, and 55.8% whereas those of the $CHCl_3$ fraction were found to be 30.8, 40.3, 49.2 and 59.8%, respectively when compared to those of the control. The ED_{50} values of the EtOH extract and $CHCl_3$ fraction were 290.8 and 222.7 mg/kg, respectively. However, the inhibition of rat paw edema offered by EtOH extract and $CHCl_3$ fraction were relatively lower than that of the positive control, indomethacin, at 10 mg/kg (69.3% inhibition at 2 h after induction). The anti-inflammatory effect of EtOH extract, $CHCl_3$ fraction and indomethacin were slightly decreased after 3 h but its effects still remained till the end of study period (5 h). In the present study, the results indicated that EtOH extract and $CHCl_3$ fraction possessed anti-inflammatory activity through the inhibition of the second phase on the release of prostaglandins and had the maximum inhibitory

Table 1
Inhibition on carrageenan-induced rat paw edema of the EtOH extract and fractions from *Boesenbergia longiflora* rhizomes.

Sample	% Inhibition at various doses at 2 h (mg/kg)						ED_{50} (mg/kg)
	0	10	50	100	200	500	
Control	0.0 ± 8.1	–	–	–	–	–	–
EtOH extract	–	–	25.3 ± 3.4	37.3 ± 7.1**	46.6 ± 3.4**	55.8 ± 7.2**	290.8
Hexane fraction	–	–	26.7 ± 5.0*	25.3 ± 2.9*	27.4 ± 6.1*	32.6 ± 3.9**	> 500
$CHCl_3$ fraction	–	–	30.8 ± 6.4*	40.3 ± 4.4**	49.2 ± 2.4**	59.8 ± 7.1**	222.7
EtOAc fraction	–	–	–	22.6 ± 1.8	32.9 ± 2.9**	34.6 ± 6.7**	> 500
H_2O fraction	–	–	–	21.0 ± 16.2	7.3 ± 14.6	14.8 ± 10.0	> 500
Indomethacin	–	69.3 ± 2.9**	–	–	–	–	–

Value represents mean ± S.E.M. ($N=6$). Significantly different from the control (vehicle, 10 ml/kg).

* $p < 0.05$.
** $p < 0.01$.

Table 2
Inhibition on NO production of the EtOH extract and its fractions from *Boesenbergia longiflora* rhizomes.

Sample	Anti-NO productions (IC_{50})		Viability of RAW264.7 cells (LC_{50})	
	μg/ml	μM	μg/ml	μM
EtOH extract	3.1 ± 0.6	–	53.3 ± 3.1	–
Hexane fraction	40.8 ± 1.8	–	≥ 100[a]	–
$CHCl_3$ fraction	5.5 ± 0.7	–	45.6 ± 1.6	–
EtOAc fraction	41.1 ± 1.0	–	–	–
H_2O fraction	60.9 ± 2.9	–	–	–
L-NA	13.5 ± 0.8	59.4 ± 3.5	–	–
Ibuprofen	14.5 ± 0.9	55.0 ± 4.3	–	–
Aspirin	16.4 ± 0.3	71.7 ± 1.5	–	–
Indomethacin	10.4 ± 0.4	29.0 ± 1.2	–	–
CAPE	1.6 ± 0.1	5.6 ± 0.4	17.1 ± 5.3	54.4 ± 12.9

Value represents mean ± S.E.M. ($N=4$). (–)=Not determined.

[a] Cytotoxic effect was observed.

effect at 2 h which was similar to that of the standard indomethacin. For the traditional remedy, the human dosage used is around 300 g of fresh rhizomes by decoction with alcohol. Thus, it is suggested that the usual dose for human is a little bit less than that used in the animal study (400 g of fresh rhizomes which is equivalent to the human dose).

3.2. *In vitro assays relevant to anti-inflammatory activity*

3.2.1. *Measurement of NO production from RAW264.7 cells*

For the *in vitro* anti-inflammatory study, LPS from Gram-negative bacteria-induced RAW264.7 macrophage cells is widely used as screening for anti-NO production activity. Over-activated macrophage by LPS is particularly well known to produce a variety of inflammatory mediators. High levels of NO induce normal cells and tissues injury by reacts with O_2^- to form peroxynitrite ($ONOO^-$), which is a powerful oxidant to normal cells function and subsequently leading to development of inflammation (Min et al., 2009; Tewtrakul et al., 2009). Using Griess reaction, NO production was determined by measuring the accumulation of nitrite in the conditioned medium of RAW264.7 cells treated with LPS. Since the EtOH extract of *Boesenbergia longiflora* showed good activity against NO release ($IC_{50}=3.1$ μg/ml), this study was then aimed to investigate the pharmacological properties and its mechanism of action. The EtOH extract was then partitioned further to obtain four fractions and tested for their NO inhibitory effects. Among these, the $CHCl_3$ fraction exhibited the most potent inhibitory effect with an IC_{50} of 5.5 μg/ml, followed by hexane, EtOAc and H_2O fractions with IC_{50} values of 40.8, 41.1 and 60.9 μg/ml, respectively (Table 2). The EtOH extract and $CHCl_3$ fraction exhibited higher effect than positive controls, L-NA (NO synthase inhibitor, $IC_{50}=13.0$ μg/ml), indomethacin ($IC_{50}=10.4$ μg/ml), ibuprofen ($IC_{50}=14.5$ μg/ml) and aspirin ($IC_{50}=16.4$ μg/ml), the clinical used non-steroidal anti-inflammatory drugs. However, EtOH extract and $CHCl_3$ fraction possessed lower activity than CAPE, an NF-κB inhibitor ($IC_{50}=1.6$ μg/ml). The results suggested that the EtOH extract and $CHCl_3$ fraction from this plant showed potent inhibitory effect on NO production in LPS stimulated RAW264.7 cells. The compounds that are responsible for this effect might be diarylheptanoids, terpenes, and/or flavonoids since these kinds of compounds are found in the $CHCl_3$ fraction of *Boesenbergia longiflora* and are mainly contained in some Zingiberaceous plants. Moreover, diarylheptanoids, diterpenes and flavonoids have been reported to show anti-inflammatory activity against NO production in our previous studies (Sae-wong et al., 2009; Kaewkroek et al., 2010).

3.2.2. *Viability assay of RAW264.7 macrophage cells*

Our results showed that the EtOH extract and its fractions significantly inhibited NO production in LPS-activated RAW264.7 cells. The cytotoxic effects were observed for EtOH extract, hexane and $CHCl_3$ fractions with LC_{50} of 53.3 μg/ml, ≥ 100 μg/ml and 45.6 μg/ml, respectively, while the EtOAc and H_2O fractions were not determined (Table 2).

3.2.3. *Detection of iNOS and COX-2 mRNA by semi-quantitative analysis of RT-PCR*

It is well known that inflammatory mediators, including NO and PGE_2 are the causes of many inflammatory diseases. NO and PGE_2 are synthesized from l-arginine and arachodonic acid via the catalytic action of the enzymes iNOS and COX-2, respectively. The mechanisms for anti-inflammatory activity of *Boesenbergia longiflora* were then studied on mRNA expression of iNOS and COX-2 genes. The results

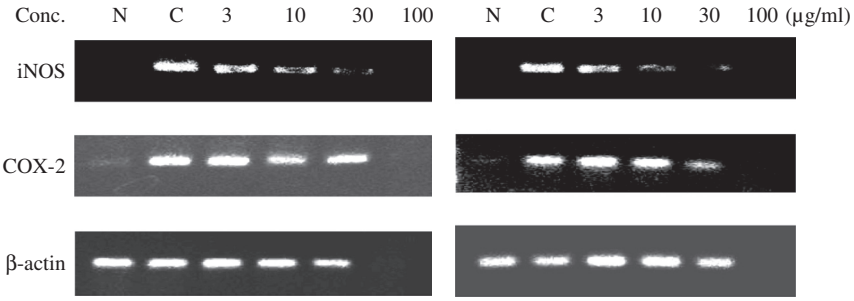

Fig. 1. Effect of the EtOH extract (A) and the $CHCl_3$ fraction (B) from *Boesenbergia longiflora* on the mRNA expressions.

Table 3
DPPH radical scavenging activity of the EtOH extract and its fractions of *B. longiflora* rhizomes.

Sample	% Inhibition against DPPH radical at various concentrations (μg/ml)							IC_{50} (μg/ml)
	1	3	10	30	100	300	500	
EtOH extract	–	−2.2 ± 1.9	0.8 ± 0.5	22.2 ± 1.1**	30.2 ± 0.4**	89.9 ± 0.9**	93.1 ± 1.0**	84.5
Hexane fraction	–	–	–	–	−2.9 ± 0.5	11.5 ± 0.3**	17.8 ± 1.9**	> 500
$CHCl_3$ fraction	–	−4.9 ± 1.2	0.2 ± 2.7	15.3 ± 0.8**	27.7 ± 1.8**	85.3 ± 1.5**	93.6 ± 0.2**	96.8
EtOAc fraction	–	7.8 ± 1.7**	29.1 ± 0.5**	89.8 ± 1.5**	94.5 ± 0.9**	95.9 ± 0.4**	94.8 ± 0.5**	17.1
H_2O fraction	–	−2.4 ± 0.5	1.2 ± 0.8	14.4 ± 0.5**	23.7 ± 1.9**	67.7 ± 1.8**	62.2 ± 1.2**	209.6
Quercetin	22.9 ± 0.4**	53.5 ± 0.2**	84.1 ± 0.6**	92.5 ± 0.2**	93.3 ± 0.5**	–	–	2.8
BHT	3.2 ± 1.0	9.5 ± 2.1**	25.9 ± 1.1**	46.4 ± 0.2**	80.9 ± 2.2**	–	–	26.7

Value represents mean ± S.E.M. (N=4). Significantly different from the control (0 μg/ml). (–)=Not determined.
** $p < 0.01$.

Table 4
Effect of the EtOH extract and its fractions of *B. longiflora* rhizomes on L929 cells viability.

Sample	% Viability of L929 cells at various concentrations (μg/ml)						
	0	0.3	1	3	10	30	100
EtOH extract	100.0 ± 1.9	98.0 ± 4.8	99.3 ± 4.6	100.8 ± 2.0	89.9 ± 5.4	56.9 ± 1.9**	11.2 ± 0.9**
Hexane fraction	100.0 ± 2.3	100.8 ± 4.0	104.4 ± 3.7	102.0 ± 5.0	119.5 ± 5.5*	112.1 ± 3.4	41.0 ± 3.6**
$CHCl_3$ fraction	100.0 ± 1.9	94.4 ± 4.4	100.8 ± 4.1	97.6 ± 8.6	91.6 ± 7.7	36.0 ± 3.1**	14.3 ± 2.1**
EtOAc fraction	100.0 ± 2.7	87.3 ± 3.2	89.5 ± 5.7	85.0 ± 5.1	85.7 ± 4.4	76.0 ± 4.9**	70.1 ± 5.3**
H_2O fraction	100.0 ± 2.7	96.3 ± 3.7	103.1 ± 6.1	98.9 ± 5.5	90.1 ± 5.5	86.6 ± 6.5	83.8 ± 5.0

Value represents mean ± S.E.M. (N=4). Significantly different from the control (0 μg/ml).
* $p < 0.05$.
** $p < 0.01$.

revealed that the EtOH extract and $CHCl_3$ fraction inhibited the expression of iNOS mRNA in a dose dependent manner, while inhibitory effect on COX-2 mRNA was partly affected (Fig. 1). In addition, the $CHCl_3$ fraction showed higher activity than the EtOH extract. However, these EtOH extract and chloroform fraction exhibited cytotoxic effect at the concentration of 100 μg/ml (Fig. 1). It is suggested that diarylheptanoids and/or flavonoids containing in *Boesenbergia longiflora* rhizomes might be responsible for the inhibition of iNOS and COX-2 genes since methoxyflavonoids isolated from *Kaempferia parviflora* (Sae-wong et al., 2011) and diarylheptanoids from *Curcuma mangga* rhizomes (Kaewkroek et al., 2010) have been reported to inhibit NO production and iNOS gene expression.

3.3. Assays relevant to wound healing

3.3.1. DPPH radical scavenging activity

The DPPH radical-scavenging activity of EtOH extract and its fractions from *Boesenbergia longiflora* rhizomes, including fractions of hexane, $CHCl_3$, EtOAc and water were shown in a dose-dependent manner (Table 3). The IC_{50} values of the EtOH extract, hexane, $CHCl_3$, EtOAc, and water fractions were 84.5, > 500, 96.8, 17.1 and 209.6 μg/ml, respectively. Of these, all fractions showed good inhibitory activity against the DPPH radical, except for the hexane fraction. In this study, the well-known antioxidant compounds, quercetin and BHT, were used as the positive controls, their IC_{50} values were found to be 2.8 and 26.7 μg/ml, respectively. The EtOAc fraction (IC_{50}=17.1 μg/ml) exhibited the scavenging activity higher than BHT (IC_{50}=26.7 μg/ml) but less than quercetin (IC_{50}=2.8 μg/ml). These results indicated that there are abundant antioxidative phytochemicals presenting in the EtOH extract and its fractions of *Boesenbergia longiflora* rhizomes, especially in the EtOAc fraction which could be flavonoids.

3.3.2. Cell proliferation and viability assay using L929 fibroblast

Fibroblast proliferation is an important step in wound healing for tissue regeneration. The proliferative effect and cytotoxicity of EtOH extract and its fractions were evaluated by MTT assay. After 24 h of treatment with EtOH extract and its fractions,

significant viability-enhancement effects were observed in L929 fibroblasts. The EtOH extract (0.3–10 μg/ml), hexane (0.3–30 μg/ml), $CHCl_3$ (0.3–10 μg/ml), EtOAc (0.3–10 μg/ml), and water fractions (0.3–100 μg/ml) produced a cell viability of more than 80% (Table 4). However, at the higher concentrations of the above concentrations showed cytotoxic effects. From our results, a better viability of fibroblast cells was observed at the lower concentrations. In addition, significant enhancement on the growth of L929 fibroblast cells was observed after treatment with the hexane fraction at 10 and 30 μg/ml ($p < 0.01$) with proliferation rate of 119 and 112%, respectively.

3.3.3. H_2O_2-induced oxidative stress

The H_2O_2-induced oxidative stress; the well-known antioxidant model was used in the present study. H_2O_2-induced oxidative damage was observed in L929 fibroblasts after 24 h of treatment with various concentrations of H_2O_2. Cell viability dramatically decreased in a dose dependent manner after treatment of 0.062, 0.125, 0.25, 0.5 and 1.0 mM H_2O_2, its LC_{50} value was 0.28 mM.

Table 5
Effect of H_2O_2 on L929 fibroblast cell viability.

% Viability of L929 cells at various concentrations of H_2O_2 (mM)					LC_{50} (mM)
0.06	0.12	0.25	0.5	1.0	
96.8 ± 2.0*	91.2 ± 1.8*	48.7 ± 2.4*	18.6 ± 2.0	15.0 ± 0.9	0.28

Value represents as mean ± S.E.M. ($N=4$).
* $p < 0.01$ as compared to 1.0 mM H_2O_2.

In addition, the effects at concentrations of 0.5 mM H_2O_2 (18.6%) and 1.0 mM H_2O_2 (15.0%) were not significantly different ($p < 0.05$), thus, 0.5 mM H_2O_2 was then selected for subsequent experiment (Table 5).

Treatment with 0.5 mM H_2O_2 for 24 h dramatically decreased cell viability to 20.0%. Different concentrations of sample pretreatment showed inhibitory effect on H_2O_2-induced cell injury with the maximum protective effect after H_2O_2 treatment. Cell viability of EtOH extract (3 μg/ml), hexane (0.3 μg/ml), $CHCl_3$ (3 μg/ml), EtOAc (3 μg/ml), and water fractions (10 μg/ml) were found to be 88.7, 95.7, 86.4, 93.0 and 88.8%, respectively (Table 6). These results were significantly different from untreated cells incubated with 0.5 mM H_2O_2 (16.2–22.6%).

The cellular aerobic metabolism, oxygen is often transformed by the mitochondria into highly reactive forms: reactive oxygen species (ROS), which are often very toxic to the cells. H_2O_2, one of the major ROS, is well known as a direct oxidant that formed hydroxyl radical to react with all components of the cell, including DNA, proteins and lipid membrane. Thus, H_2O_2 induced oxidative stress is a useful method for gaining the endogenous antioxidant activity. While, the DPPH assay showed effectively neutralize harmful extracellular free radicals (Jitsanong et al., 2011). The results suggest that EtOH extract and its fractions, except for the hexane fraction, might provide a protective effect by acting as extracellular antioxidants. Interestingly, the hexane fraction that did not show anti-oxidant activity by DPPH assay significantly inhibited the oxidative stress induced cell injury by H_2O_2. Therefore, the ability of hexane fraction may protect cells from oxidative injury in an intracellular environment and the compounds showing this effect should be non-polar than those from the $CHCl_3$ fraction.

Table 6
Protective effect of the EtOH extract and its fractions of *B. longiflora* rhizomes on 0.5 mM H_2O_2-induced L929 fibroblast death.

Sample	% Viability of L929 cells at various concentrations (μg/ml)					
	Control	H_2O_2	0.3	1	3	10
EtOH extract	100.0 ± 2.4	20.0 ± 0.7	76.6 ± 4.6*#	87.7 ± 5.2#	88.7 ± 6.3#	77.7 ± 2.6*#
Hexane fraction	100.0 ± 2.3	20.6 ± 2.4	95.7 ± 0.3#	88.8 ± 3.1*#	80.8 ± 2.0*#	66.5 ± 1.3*#
$CHCl_3$ fraction	100.0 ± 2.7	17.1 ± 0.3	80.2 ± 1.8*#	83.8 ± 2.6*#	86.4 ± 3.2*#	80.0 ± 2.4*#
EtOAc fraction	100.0 ± 4.4	16.2 ± 0.5	93.6 ± 1.7#	88.8 ± 1.7*#	93.0 ± 0.9#	78.3 ± 1.0*#
H_2O fraction	100.0 ± 4.9	22.6 ± 0.8	69.2 ± 1.7*#	73.3 ± 0.5*#	74.8 ± 1.8*#	88.8 ± 1.3*#

Value are expressed as mean ± S.E.M. ($N=4$).
* $p < 0.01$ compared with control (0 μg/ml).
# $p < 0.01$ compared with 0.5 mM H_2O_2 treated cells.

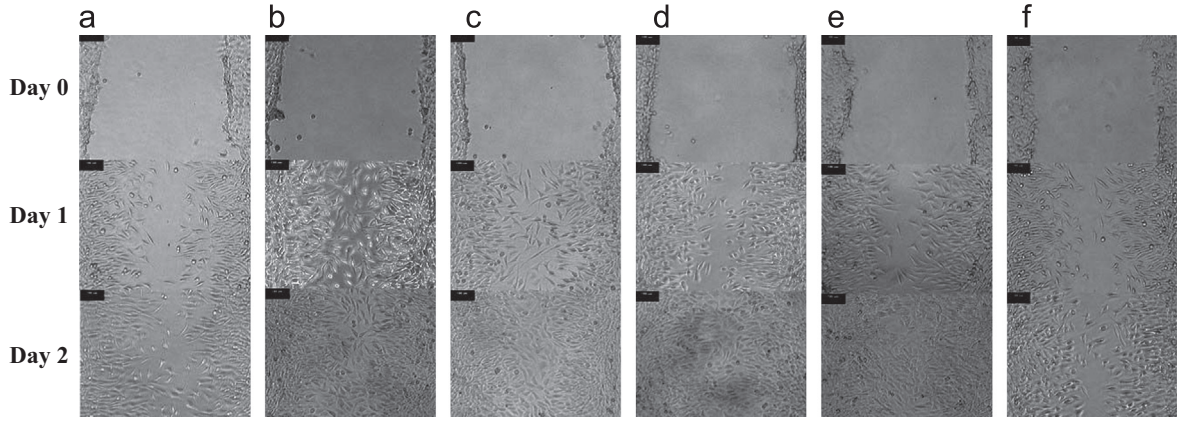

Fig. 2. Effect of the EtOH extract and its fractions from *Boesenbergia longiflora* rhizomes on fibroblast L929 migration. Monolayer of L929 were scratched horizontally with P100 pipette tips. Images were captured at day 0 and then treated with 3.0 μg/ml EtOH extract (b), 10.0 μg/ml hexane fraction (c), 3.0 μg/ml $CHCl_3$ fraction (d), 10.0 μg/ml EtOAc fraction (e), 10.0 μg/ml H_2O fraction (f) and control (a) without treatment. Another set of images were captured at day 1 and 2 after incubation. Quantitative analysis of the migration rate was quantified by computing software.

Table 7
Effect of the EtOH extract and its fractions of *B. longiflora* rhizomes on *in vitro* scratch assay using fibroblast L929.

Sample	Dose	Length between the scratch (μm)			% Migration rate of cells	
	(μg/ml)	Day 0	Day 1	Day 2	Day 1	Day 2
Control	–	582.1 ± 2.3	352.9 ± 11.3*	267.9 ± 10.7*	39.4 ± 2.0	54.0 ± 1.8
EtOH extract	3	645.7 ± 4.3	196.4 ± 7.0*	98.2 ± 7.4*	66.3 ± 1.2*	83.1 ± 1.3*
Hexane fraction	10	508.9 ± 9.7	139.3 ± 8.9*	0.0*	76.1 ± 1.5*	100.0*
$CHCl_3$ fraction	3	614.3 ± 8.5	132.1 ± 11.7*	0.0*	77.3 ± 2.0*	100.0*
EtOAc fraction	10	579.3 ± 7.6	157.1 ± 7.1*	0.0*	73.0 ± 1.2*	100.0*
H_2O fraction	10	560.7 ± 5.9	333.9 ± 8.5*	226.8 ± 6.6*	42.6 ± 1.5	61.0 ± 1.1*

Value represents mean ± S.E.M. (N=4). Significantly different from the control. (–)=Not determined.
* $p < 0.01$.

Table 8
Collagen type-I production in L929 cells when treated with the EtOH extract and its fractions of *B. longiflora* rhizomes.

Sample	Collagen production at various concentrations (μg/ml)				
	0	0.3	1	3	10
Control	60.8 ± 0.7	–	–	–	–
EtOH extract		63.8 ± 1.4	169.6 ± 2.8*	187.9 ± 1.7*	93.8 ± 2.6*
Hexane fraction		22.5 ± 2.5*	94.2 ± 1.2*	117.1 ± 1.0*	187.5 ± 3.3*
$CHCl_3$ fraction		19.2 ± 2.0*	97.1 ± 2.4*	187.5 ± 2.4*	85.0 ± 3.4*
EtOAc fraction		< 2.5	23.8 ± 1.8*	75.8 ± 1.8*	112.1 ± 1.4*
H_2O fraction		< 2.5	< 2.5	< 2.5	5.8 ± 2.4*

Value represents mean ± S.E.M. (N=4). Significantly different from the control. (–)=Not determined.
* $p < 0.01$.

3.3.4. *Effect of Boesenbergia longiflora extract on migration of L929 cells*

In the present study, the EtOH extract and its fractions of *Boesenbergia longiflora* were determined on the rate of L929 migration using the scratch assay. Scratch assay is a useful method for gaining an insight into the potential of an extract or its fractions to repair injured dermis. This assay is commonly used to study cell migration *in vitro* by creation of an artificial gap on a confluent cell monolayer with a pipette tip (Balekar et al., 2012). The cellular proliferation and migration of fibroblast cells on each edge of the gaps move toward to close the scratch area until new cell–cell contacts was studied on days 0, 1 and 2. As shown in Fig. 2, the presence of EtOH extract (3 μg/ml), hexane (10 μg/ml), $CHCl_3$ (3 μg/ml), EtOAc (3 μg/ml), and water fractions (10 μg/ml) caused an increased migration of L929 fibroblasts significantly ($p < 0.05$) on day 1 by 66.3, 76.1, 77.3, 73.0 and 42.6%, respectively. In addition, more significant increases in L929 fibroblasts migration were observed on day 2 by 83.1, 100, 100, 100 and 61.0%, respectively (Table 7). The results demonstrated that there was a significant enhanced migration effect of the EtOH extract and its fractions when compared with the control group.

3.3.5. *Effects on soluble collagen production from fibroblast L929*

Fibroblasts synthesized collagens are the most abundant family of protein in the body that provide strength and integrity to all tissues and also play a vital role in wound healing (Enoch and Leaper, 2008). Collagen type-I is the main structural component of extracellular matrix, skin and newly healed wounds. High level of skin collagen was shown to be involved in stimulating improvements on skin elasticity (Balekar et al., 2012). The study on the effect of EtOH extract and its fractions on the type-I collagen production by fibroblast was performed by using Sircol collagen assay kit. The results showed that collagen type I production in L929 cells increased significantly after treatment with EtOH extract and its fractions at various concentrations (Table 8). The amount of collagen produced by the cells was between 50 and 200 μg/ml. The levels of collagen in the culture supernatant medium of fibroblasts after treatments with samples for 48 h were increased as compared to that of the control. However, there was no detectable collagen generated by fibroblasts after activation by H_2O fraction.

In this study, our results revealed that the wound healing property of the EtOH extract and $CHCl_3$ fraction of *Boesenbergia longiflora* is involved in the proliferation and migration of fibroblast cells, and stimulation of collagen production. Moreover, they also possess anti-inflammatory effect as well as anti-oxidant activity which are important factors for wound healing enhancement.

4. Conclusion

The present study supports the traditional use of *Boesenbergia longiflora* for treatment of inflammatory bowel disease, ulcerative colitis, aphthous ulcer and abscess which is related to the wound healing potential. The EtOH extract and $CHCl_3$ fraction of *Boesenbergia longiflora* significantly enhanced L929 fibroblast migration and collagen production. It also revealed that EtOH extract and $CHCl_3$ fraction inhibited the NO production in macrophages, suggesting their potential as anti-inflammatory agents. They also found to suppress the mRNA expression of iNOS and COX-2 genes in dose-dependent manners. In addition, they are shown to be potent for anti-inflammation *in vivo* by lowering the rat paw edema induced by carrageenan. From the phytochemical study, the $CHCl_3$ fraction of *Boesenbergia longiflora* was found to contain diarylheptanoids, flavonoids and terpenes. These groups of compounds might be responsible for the wound healing property of this plant. The isolation and structure elucidation of compounds responsible for the wound healing effect of *Boesenbergia longiflora* are now in progress.

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